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IMMUNOGLOBULINS
AND
CROSSED
IMMUNOELECTROPHORESIS

by

ANDERS O. GRUBB

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by

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This thesis concerns the subjects of the following communications referred to in the text by their Roman numerals:



- I. Quantitation of Immunoglobulin G by Electrophoresis in Agarose Gel Containing Antibodies. *Scand. J. Clin. Lab. Invest.* 26, 249 (1970)
- II. Quantitative Determination of the Distribution of the Specific Antibodies of Antisera Subjected to Gel Electrophoresis. *Immunochemistry* 9, 635 (1972)
- III. Analysis of the Immunochemical Relationship between Antigens by Electrophoresis in Agarose Gels Containing Antibodies. *Scand. J. Clin. Lab. Invest.* 29, Suppl. 124, 59 (1972)
- IV. Immunochemical Quantitation of IgG: Influences of the Antiserum and of the Antigenic Population. *Scand. J. Clin. Lab. Invest.* 31, 465 (1973)
- V. Preparation of Electroendosmosis-Free Agarose Gel and Exemplification of Its Use in Crossed Immuno-electrophoresis. *Anal. Biochem.* 55, 582 (1973)
- VI. Crossed Immuno-electrophoresis and Electroimmunoassay of IgM. *J. Immunol.* 112 (1974). *In press.*
- VII. Crossed Immuno-electrophoresis and Electroimmunoassay of IgG. *J. Immunol.* 113 (1974). *In press.*

L. James Brown has revised the language of the communications and of the thesis

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PART ONE

IMMUNOCHEMICAL QUANTITATION OF IMMUNOGLOBULINS

The use of immunochemical methods for determining concentrations of substances in various fluids is extensive and is still increasing rapidly. This is not only because such methods are simple, quick and cheap, but also because the concentration of certain substances can at present be determined only with such methods.

It is often difficult to achieve a high degree of accuracy with such methods. This applies in particular to quantitation of substances made up of heterogenous molecular populations, since different molecular populations of such substances may differ considerably from one another in composition. Such substances are common. Examples are haptoglobin and the various immunoglobulin classes. This part of the thesis concerns problems associated with the achievement of a high degree of accuracy of immunochemical quantitation of heterogenous molecular populations.

Immunochemical quantitation is always based on the production of an antiserum specific for the substance (the molecular population) to be quantitated. The reaction between the antiserum and a sample with an unknown content of the substance to be quantitated (test sample) is afterwards compared by one method or another with the reactions between the antiserum and several standard samples with a varying known content of the substance. In view of this general procedure for immunochemical quantitation problems bearing on the achievement of a high degree of accuracy of immunochemical quantitation of substances with heterogeneous molecular populations appear to fall into the following three groups. It should, however, be emphasised that the problems encountered in any one of the three groups are only rarely independent

of those in the other groups.

1. Problems bearing on the difference between the molecular population in the test sample and that in the standard sample.

2. Problems related to the type of antiserum.

3. Problems, related to the choice of method used for comparing the reaction between, on one hand, the antiserum and the test sample and, on the other hand, the antiserum and the standard sample.

In the following elucidation of these problems, the immunochemical quantitation of immunoglobulins will be used as an example. This example was chosen, first, because such quantitation is extremely common in research and routine medical work with the use of many different types of methods and antisera and, second, because the immunoglobulins are examples of extremely heterogeneous molecular populations.

Problems bearing on the difference between the molecular population in the test sample and that in the standard sample

The composition of a molecular population of an immunoglobulin in a test sample and in a standard sample used in an immunochemical quantitation may be identical with, or differ widely from, one another. In the former case good accuracy can be achieved by immunochemical quantitation without any special claims on the choice of method or antiserum. In the latter case, however, special demands must be placed on the choice of antiserum and method if a high degree of accuracy is to be obtained. This is obvious from some investigations where purified

IgG populations differing from one another in composition were quantitated by immunochemical methods and by physico-chemical methods (1,IV). The investigations showed that the degree of accuracy tended to vary inversely with the magnitude of the difference in composition between the IgG population of the test sample and that of the standard sample.

The observations set forth above imply that if different laboratories use different standard samples (with immunoglobulin populations of different composition) the values found at different laboratories for the immunoglobulin content of one and the same test sample will differ even if they all use the same antiserum and the same method. Now, if different laboratories use different standard samples (calibrated in different ways against purified immunoglobulin populations) different methods and different antisera for immunochemical quantitation of the immunoglobulins in a given test sample, the interlaboratory variation of the results will be wide. This has clearly been shown by Rowe *et al.* (2), who also demonstrated that if one and the same standard sample is used at different laboratories the interlaboratory variation will not be so wide (3). This finding induced WHO recently to introduce an International Reference Preparation of Human Immunoglobulins IgG, IgA and IgM (4) and to recommend that immunoglobulins be measured in international units with the use of the International Reference Preparation as standard sample. Similar reference preparations are available for measuring IgD and IgE (5,6). The international reference preparations of WHO consist of pooled plasma obtained from healthy adults.

The compositions of the normal polyclonal immunoglobulin populations in WHO's reference preparations may differ markedly from those of the immunoglobulin populations of a test sample. For instance, two IgG-

populations may differ from each other in respect of, among other things, their subclass composition, their κ/λ -quotient, their Gm-composition, their subgroup composition etc. A monoclonal IgG-population which commonly occurs in plasma from persons with multiple myeloma, for example, differs markedly in all these respects from the normal IgG-population. The immunochemical quantitation of such monoclonal IgG-populations therefore often gives very erroneous results. This is true whether such methods are used as single radial immunodiffusion (1,IV) or electroimmunoassay (7), which are based on the quantitative precipitin reaction, or methods based on inhibition of the primary antigen-antibody interaction, such as Farr's ammonium sulphate method (8,9) solid phase radioimmunoassays (1) or inhibition of passive haemagglutination (10). Similar difficulties are encountered in immunochemical quantitation of monoclonal populations of the remaining immunoglobulin classes (A,M,D,E) even though these difficulties have only occasionally been reported (11,12). These difficulties explain the reluctance to use immunochemical methods in the quantitation of monoclonal immunoglobulin populations and to give preference to physico-chemical methods in which the binding of dye to the monoclonal populations is measured, even though also the latter methods are often imprecise and inaccurate (13).

In the immunochemical quantitation of immunoglobulins the differences between the immunoglobulin populations in the test sample and those in the standard sample are rarely so large as between a monoclonal and a normal polyclonal population. On the other hand, the two populations are rarely identical. As a rule, the differences between the populations lie somewhere between these two extremes and thus comprise major or minor differences in subclass composition (14, VII), κ/λ -quotient, Gm-composition and subgroup composition etc. The accuracy of an immunochemical quantitation will therefore vary somewhat

from one test sample to another. The range of such variation is determined by the type of antiserum and method used and by the nature and size of the differences between the test samples and the standard samples.

A special aspect of the difference between the immunoglobulin population in a test sample and that in a standard sample is that concerning the size distribution of the molecules within an immunoglobulin class. An IgA-population is often made up of monomers, dimers, higher polymers and secretory IgA, for example. An IgM-population is usually made up of 7S and 19S IgM. Free light immunoglobulin chains occur generally as monomers, dimers and tetramers (15). Complex formation with participation of the immunoglobulins is not uncommon (16,VI). If the size distribution of the molecules within an immunoglobulin population in the test sample differs from that in the standard sample, the accuracy of some immunochemical methods, such as single radial immunodiffusion, will be poor (17,18,19). Other methods seem to be more independent of the size distribution of the molecules within an immunoglobulin class (20), but, as far as I know, no systematic investigations have been made to find out in what way the results of different methods for immunochemical quantitation are influenced by the size distribution of the immunoglobulin molecules.

One way of improving poor accuracy of a method for quantitation of an immunoglobulin, in which the poor accuracy is due to differences in the molecular size distribution of the immunoglobulin in the test sample and in the standard sample, would be to render the size distributions of the two samples identical. This would probably sometimes be possible by simple reduction (VI) and/or by allowing the immune reactions to take place in slightly dissociating media. But in some cases correct immunochemical quantitation would surely require laborious methods with fractionation of an immunoglobulin popula-

tion into its different molecular weight classes and separate quantitation of them with the use of several standard samples with immunoglobulin populations of corresponding molecular weight classes.

Problems related to the type of antiserum

In immunochemical quantitation the degree of accuracy of a determination will often depend to a large extent on the choice of antiserum. This dependence increases with the difference between the immunoglobulin population in the test sample and in the standard sample, at least as far as some immunochemical methods are concerned. This has been clearly shown in an investigation (IV) where purified IgG M-components of different subclasses and light chain types were quantitated with single radial immunodiffusion with the use of different types of specific anti-IgG-antisera. The standard sample contained a polyclonal IgG-population. The various IgG-populations were also quantitated physico-chemically. The results showed that the accuracy of the immunochemical quantitation of a monoclonal IgG-population varied widely, first, with the type of antiserum used and, second, with the subclass of the IgG-population for some, but not for all, types of antisera. The quantitation of a polyclonal IgG-population with different antisera gave better accuracy than the quantitation of monoclonal IgG-populations, irrespective of the type of antiserum used.

Observations in line with those set forth above have also been reported by Morell *et al.* (1), who compared normal polyclonal IgG with purified monoclonal IgG-populations with single radial immunodiffusion and with solid phase radioimmunoassay with the use of different types of anti-IgG-antisera.

It should be stressed that the difference between

different antisera may be very large even if all of them are monospecific for an immunoglobulin class. Antisera specific for IgG, for example, may contain a varying mixture of antibodies to a) antigenic determinants common to all IgG-molecules, b) subclass-specific antigenic determinants, c) antigenic determinants shared by some, but not by all, IgG-subclasses, d) different subgroup-specific antigenic determinants, e) different idiotypic antigenic determinants etc. From a theoretical point of view an anti-IgG-antiserum containing only antibodies to antigenic determinants common to all IgG-molecules, should give the highest degree of accuracy irrespective of the method of immunochemical quantitation. This is because such an antiserum would not recognize any antigenic differences between two different IgG-populations no matter how different they may be. This holds also for antisera to other immunoglobulin classes (and free κ - and λ - chains). How such ideal antisera could be produced and how an antiserum should be tested for it to be recognizable as ideal, has yet to be described, however. But empirical support (IV) has been obtained that antisera against the comparatively homogeneous Fc-fragments of an immunoglobulin give much higher degree of accuracy than do many other types of antisera. It is also widely recognized that antisera raised against unfragmented M-components give poor accuracy in immunochemical quantitation of the corresponding immunoglobulin class (21,22). Relatively simple methods for preparing Fc-fragments of IgG (23), IgM (24), IgD (25) and IgE (26) have been available for some time and has recently been described for the preparation of Fc-fragments of IgA (27) and the constant halves of light immunoglobulin chains (28). Antisera against such comparatively homogeneous fragments of immunoglobulins should therefore now be more widely used at immunochemical quan-

tititation of immunoglobulins.

Problems, related to the choice of method used for comparing the reaction between, on one hand, the antiserum and the test sample and, on the other hand, the antiserum and the standard sample

It has been clearly shown by Polmar *et al.* (29) that the result of immunoglobulin quantitation can vary widely from method to method even if the same antiserum and standard samples are used in all the methods. In their investigation three techniques for radioimmunoassay (Sephadex-bound antibody, bromoacetylcellulose-bound antibody and double antibody) of IgE were used in the examination of a large number of test samples. The results obtained with some samples varied widely from one method to the other despite the use of the same antiserum and standard samples. The results suggested the occurrence of non-specific inhibition of the binding of IgE to the insolubilised anti-IgE-antibodies in the two solid phase radioimmunoassays though the conventional claims placed on the specificity of a radioimmunoassay (specificity of antibodies, parallelism of dose-response curves for unknowns and standards, lack of inhibition by purified relevantly selected proteins) were filled by both methods. Such a non-specific inhibition of antigen-binding to antibodies is less probable in radioimmunoassays, in which the double antibody technique is used. But it cannot be excluded, not even in this measuring system, because some of the test samples may contain antibodies against the immunoglobulins of the immunized animal and thereby interfere with the antigen-binding in any type of radioimmunoassay. Autoantibodies against the antigen whose content is to be determined may also seriously disturb some radioimmunoassays. Human autoantibodies to

IgE, which interfere with a solid phase radioimmunoassay have been described by Lundkvist (30), for example.

Morell *et al.* (1) quantitated a number of purified IgG M-components with both a solid phase radioimmunoassay and with a gel precipitation technique (single radial immunodiffusion; 31, 32). Though the same antisera and standard samples were used in both methods, the values obtained were sometimes widely different. The differences between the values obtained with the two methods did not appear to fall into a readily discernible systematic pattern. The causes of the differences are probably related to the fact that the two methods measure the antigen-antibody interaction in two different ways. In the solid phase radioimmunoassay it is the degree of inhibition of the primary antigen-antibody interaction that is measured and in the single radial immunodiffusion it is the secondary precipitate formation. These two measuring systems are probably influenced in different ways by the content of non-precipitating antibodies in an antiserum, for example.

The values found for immunoglobulin concentrations may be too high not only in different types of radioimmunoassays owing to non-specific inhibition of the primary antigen-antibody interaction, but also in most methods based on the quantitative precipitin reaction (33) owing to the formation of precipitates other than immunoprecipitates. Such methods are, among others, those which use immunoprecipitation in gels, *e.g.* single radial immunodiffusion (31,32), single linear diffusion *ad modum* Oudin (34), double linear diffusion according to Preer (35) or Ouchterlony (36) or Wieme and Veys (37), electroimmunoassay (7,38,39,VI,VII) and counterimmuno-electrophoresis (40). Those precipitates which in the above-mentioned methods can be erroneously interpreted as immunoprecipitates may be produced by interaction

between soluble substances in the test samples and antisera (*e.g.* complement factors and ribonucleic acids; 41,42,43) as well as by interaction between the stabilizing medium and soluble substances in the test samples (44).

Just as test sample antibodies against the immunoglobulins of the immunized animal can interfere with the results of radioimmunoassays, they can also disturb immunochemical quantitation based on the quantitative precipitin reaction (45).

Summary of part one

The cause of the poor accuracy of many immunochemical methods for determinations of immunoglobulins is generally the differences between the immunoglobulin populations in the test samples and those in the standard samples. The accuracy can be improved and the interlaboratory variation can be reduced by observance of the following recommendations.

1. WHO's international reference preparations of human immunoglobulins should be used as standard samples. Other preparations containing immunoglobulin populations resembling those of WHO and whose immunoglobulin content has been determined with WHO's preparations as primary standard samples may also be used as working standard samples.

2. Differences in the molecular weight distribution of the immunoglobulin populations in the test samples and in the standard samples should, when possible, be eliminated before the determination proper.

3. An antiserum and a method should be used which recognizes as few differences as possible between the

immunoglobulin populations of the test samples and standard samples.

As for the choice of antisera, antisera against the relatively homogeneous Fc-fragments of immunoglobulins appear to give better accuracy than most other types of antisera used today. Further investigations are necessary to ascertain whether new types of antisera can give still better accuracy.

As for the choice of method, the decision whether any particular method gives better accuracy than another must abide further research.

PART TWO

IMMUNOGLOBULINS AS ANTIBODIES IN CROSSED IMMUNO- ELECTROPHORESIS

Crossed immunoelectrophoresis (46,47,48,49) has a high resolving power and gives quantitative information. This has made the technique extremely valuable in the investigation of heterogeneity, hereditary polymorphism, formation of complexes and fragmentation of antigens. The technique consists of two equally important phases, *viz.* the separation of the antigens and the reaction of antigens with antibodies in a gel-buffer system in an electric field. This part of the thesis concerns the second phase with special reference to those properties of the antibodies that are necessary for obtaining good results. It should be emphasized that these properties are the same as those required for obtaining good results of the electroimmunoassay.

To facilitate apprehension of the factors essential for a good result, a schema of the sequence of events in ideal crossed immunoelectrophoresis is given below.

A gel strip with separated antigens of different sizes and electrophoretic mobilities is placed in a slit of corresponding size in a gel containing antibodies against one or more of the antigens. The strip of gel with the antigens and the gel containing antibodies may be of the same or of different type. Also the buffers in the two gels may be identical or different. An electric field is applied across the gels perpendicularly to the longitudinal axis of the gel strip. The antigens thereby migrate into the antibody-containing gel and are bound to the antibodies, whose mobility is zero in the gel-buffer system used. The first small soluble antigen-antibody complexes that form in the presence of antigen excess migrate, without sterical hindrance, in the same direc-

tion in the gel-buffer system as the free antigen. As the electrophoresis proceeds the antigen excess decreases, the antigen-antibody complexes increase in size and are eventually precipitated. The area enclosed by the precipitation line formed and the edge of the gel strip is directly proportional to the total amount of antigen.

With reference to the aforementioned schema it would appear that if good results are to be obtained, careful attention must be given to the following points.

1. The separation of antigen.
2. The specificity of the antiserum.
3. The co-occurrence of precipitates other than immunoprecipitates.
4. The migration of the antibodies.
5. The valence of the antibodies.
6. The size of the antibodies.
7. The avidity (affinity) of the antibodies and the precipitation tendency of the antigen-antibody complexes.
8. The gel type used for the antigen-containing gel strip and for the antibody-containing gel.
9. The buffer system.
10. The visualization of the pattern of immunoprecipitation.

1. Separation of antigen

That separation of antigens is important for obtaining a good result of crossed immunoelectrophoresis is self-evident, but since the separation does not involve any problems peculiar to crossed immunoelectrophoresis it will not be discussed in this treatise. It might, however, be mentioned that those methods used for separating antigens are isoelectric focusing in polyacrylamide gel (50,51,52,53,V) and usually, different types of zone-electrophoresis.

2. Specificity of the antiserum

The specificity of the antiserum used must be well-defined if interpretable findings are to be obtained in immunochemical procedures. This is true also for crossed immunoelectrophoresis, but since this method sometimes reveals smaller differences between two antigens or two antisera than do other methods (54,55,III) it may sometimes be necessary to test the specificity of an antiserum directly in crossed immunoelectrophoresis instead of in a technically simpler test system. Several modifications of crossed immunoelectrophoresis suitable for testing the specificity (or titre) of an antiserum have been reported (54,55,56,57,58,59,60,III).

3. Co-occurrence of precipitates other than immuno- precipitates

The pattern of precipitation obtained on crossed immunoelectrophoresis can be disturbed by the co-occurrence of precipitates other than immunoprecipitates. Such precipitates include those formed by interaction between soluble substances (*e.g.* ribonucleic acids and complement factors) in the test sample and in the antiserum

(41,42,43) as well as those formed by interaction between the stabilizing medium (agarose) and soluble substances in the test sample (44,61) as well as those precipitates, if any, formed by activation of the coagulation system in a plasma sample (62). But the formation of such unspecific precipitates can often be easily prevented. The use of purified antibodies instead of unfractionated antiserum will usually prevent the formation of the first-mentioned type of unspecific precipitates, for example, while the use of purified agarose will prevent the formation of precipitates by adsorption to the agarose gel (61). The activation of the coagulation system in a plasma sample can easily be prevented by the use of buffers containing EDTA or by separating the coagulation factors by electrophoresis in an antiserum-free gel before any immunoprecipitation can take place in an antiserum-containing gel (62).

4. Migration of the antibodies

It is well-known that the result of crossed immunoelectrophoresis is strongly influenced by the migration of the antibodies during the immunoelectrophoretic phase (46,63,64,II). In a work (II) where antibody-populations with homogenous electrophoretic mobilities were used, it was shown that precipitation lines formed by antibodies and antigens migrating in the same direction are diffuse, while those formed by immobile antibodies and (mobile) antigens are well defined, as are those formed by antibodies and antigens migrating towards each other. It was further shown that antibodies migrating in the same direction as the antigen produced precipitation patterns which overestimated the amounts of antigens. When the antibodies and antigens migrated towards each other the precipitation pattern underestimated the amounts of antigens.

Migration of the antibodies during the immuno-electrophoretic phase of crossed immunoelectrophoresis depends mainly on the electrophoretic mobility of the antibodies and on the electroendosmosis of the agarose gel.

The electrophoretic mobility of the native antibodies varies with the species of the animal in which the antiserum is raised (64,65) and with the charge of the antigen used for immunization (66,67,I). If fractionated antisera are to be used in crossed immunoelectrophoresis it should be borne in mind that several methods for fractionation are accompanied by an enrichment of cathodal antibodies (68,I).

The electroendosmosis of the antibody-containing agarose gel may vary widely not only with the brand, but also with the batch, of agarose used (69).

As mentioned in the introduction, the antiserum most suitable for use in crossed immunoelectrophoresis is the one in which the migration of the antibodies is as slow as possible. The production of such antisera has recently been made possible by the development of a method for quantitative determination of the distribution of the antibodies of antisera subjected to gel electrophoresis (II) together with methods for raising (VI,VII) or lowering (70) the isoelectric points of antibodies without appreciably affecting their immunoreactivity.

The electroendosmosis of the agarose-gel can also be adjusted within wide limits by additions of agar (with high cathodal electroendosmosis) or of an agarose-derivative with anodal electroendosmosis (V). Further information on this point is given in a subsequent section. (See subheading 8!).

5. Valence of the antibodies

Hyperimmune mammalian sera are mostly used in crossed

immuno-electrophoresis and thus antibody populations consisting almost entirely of divalent 7S antibodies. Such divalent antibodies are presumably the most suitable type for use in crossed immuno-electrophoresis. Antibodies with only one combining site (*e.g.* Fab-fragments produced by papain-digestion of an antibody-population of divalent 7S antibodies) are not suitable for use in crossed immuno-electrophoresis because they do not form precipitates. As far as I know, dekaivalent (IgM) antibodies have never been used in crossed immuno-electrophoresis. They are probably less suitable than divalent antibodies because of the largeness of their size (See subheading 6!).

6. Size of the antibodies

Good results of crossed immuno-electrophoresis requires that the antigen-antibody complexes formed in antigen excess migrate, without sterical hindrance, in the same direction as the free antigen. Therefore, the smaller the antibodies the better, because the smaller the antigen-antibody complexes formed in antigen excess the less the sterical hindrance to their migration. Further, the larger the antigen part of the complexes the closer the agreement between the migration of the complexes and that of the free antigen also when the gel does not exhibit any molecular sieving properties.

It would thus appear that 7S antibodies are more suitable for crossed immuno-electrophoresis than 19S antibodies although this has not yet been experimentally verified. It would also seem that fragments of 7S antibodies are more suitable than the native antibodies as far as analysis of small, slowly migrating antigens are concerned provided, of course, that the fragments can still form precipitates with the antigen. Conceivable fragments of this type are the $F(ab')_2$ -fragments produced by pepsin-digestion.

7. Avidity^Δ (affinity) of the antibodies and precipitation tendency of the antigen-antibody complexes

The antigen-antibody complexes formed in antigen excess on crossed immunoelectrophoresis may be either insoluble or soluble in the gel-buffer system used. In the former case the precipitation will be spread over a diffusely outlined area. In the latter case well defined arcuate precipitation lines form, provided that the complexes are soluble in the large antigen excess at the beginning of the immunoelectrophoretic phase but not on the diminution of the antigen excess occurring on progression of the electrophoresis. If the complexes are soluble in both antigen excess and in antibody excess as well as in the equivalence zone, no precipitate will form.

The results of crossed immunoelectrophoresis are best if the precipitation tendency of the antigen-antibody complexes is such as to produce well defined lines. The precipitation tendency is determined mainly by properties of the antibodies, the gel-buffer system, the antigen, and the voltage used for the immunoelectrophoretic phase.

^Δ

The affinity of an antibody is defined as the binding constant between an antigenic determinant and the antibody but since this constant can be determined only for haptens the term avidity is used to denote the tendency of an antibody to form a stable complex with a macromolecular antigen. The avidity is therefore determined not only by the affinity of the antibody for an individual antigenic determinant on the macromolecule but also by other factors, such as the electric charge or molecular shape of the antigen.

The antibody property chiefly influencing the precipitation tendency of the antigen-antibody complexes is the avidity (71). The higher the avidity the larger the complexes and thereby the stronger their precipitation tendency. If the avidity of the antibodies is low, the complexes formed may be so small as to produce no demonstrable precipitates, not even in the equivalence zone. The avidity of the antibodies depends on, among other things, the immunization procedure (72), in which injections of antigens with complete Freund's adjuvant seem to give rise to antibodies of unusually high avidity. The antibody avidity is sometimes irreversibly reduced if the antibodies are treated with buffers of extreme pH or containing chaotropic ions in high concentration. Such treatment is often used when antibodies are purified by procedures involving the use of immunosorbents.

Other antibody properties influencing immunoprecipitation are those which decide whether antibodies in excess will dissolve an already formed immunoprecipitate. Excess of antibodies from *inter alia* man, rabbit, goat, pig, dog, guineapig, rat and chicken will generally not dissolve an already formed immunoprecipitate, while an excess of equine antibodies often will (73). This means that equine antibodies are sometimes unsuitable for use in crossed immunoelectrophoresis (74).

The precipitation tendency of the antigen-antibody complexes varies with the composition of the gel-buffer system. If certain water-soluble polymers, such as dextran or polyethylene glycol, be added to the gel-buffer system it will markedly increase the precipitation tendency of the complexes (71,75,76,77). Such polymers may be used in crossed immunoelectrophoresis to enhance the sensitivity of the method or to stimulate the formation of precipitates in antigen-antibody systems in which otherwise no precipitates are formed. Such polymers can also be used to prevent the otherwise occasional

electrophoretic migration of some immunoprecipitates containing small antigens (39). Inclusion of a detergent in the gel-buffer system reduces the precipitation tendency of the complexes and at the same time enhances the solubility of many antigens. This can be utilized to make possible a study of antigens of poor solubility *e.g.* membrane antigens, by crossed immunoelectrophoresis (78). In such studies it is important to adjust the concentration of the detergent in such a way as to prevent non-specific precipitation of the antigens of poor solubility but at the same time permit some precipitation of immune complexes (79). Other obvious properties of the gel-buffer system which influence the precipitation tendency of the immune complexes are pH, ionic strength, temperature etc.

The characteristics of the antigen which most profoundly influences the precipitation tendency of the immune complexes are the size of the antigen and its number of antigenic determinants. These two characteristics often, but not always, vary with one another. Large antigens carrying many determinants (*e.g.* 19S IgM and fibrinogen) give rise to immune complexes with a strong precipitation tendency and therefore sometimes to precipitation despite moderate antigen excess. This means that when such antigens are studied by crossed immunoelectrophoresis precipitates appear as areas instead of as well defined precipitation lines (39,80). Small antigens carrying few determinants give rise to immune complexes with a low precipitation tendency, sometimes so low that no precipitation occurs even in the equivalence zone or in the zone of antibody excess. Such small antigens can therefore often not be studied by crossed immunoelectrophoresis. Antigens of intermediate sizes usually give rise to immune complexes that are precipitated as well defined lines and can therefore be studied with advantage by this method.

The precipitation of the immune complexes varies also with the voltage used. As a rule, especially when large

antigens are studied, high voltage gives rise to diffuse precipitation areas, while low voltage gives well defined precipitation lines (39,VII). This might be because the equilibrium between the immune complexes formed early in the process and the free antigen is disturbed by the rapid separation of the free antigen from the complexes in an electric field of high voltage. For the maintenance of this equilibrium is important to keep the small size and high solubility of the complexes formed early in the process.

8. Gel type used for the antigen-containing gel strip and for the antibody-containing gel

The separation of antigen (by zone electrophoresis or by isoelectric focusing) in the first phase of crossed immunoelectrophoresis can be performed with the use of several different stabilizing media such as agarose gel, starch gel and polyacrylamide gel (46,47,48,49,50,51,52, 53,81,82,V). Only agarose or a mixture of agarose and polyacrylamide (52) has been used as stabilizing medium in the second phase of the procedure. In order to keep the separation of the antigen in the gel strip intact during the second phase the intimate contact between the gel strip and the antibody-containing agarose must not be disturbed. Such disturbances are, however, apt to occur when stabilizing media other than agarose are used in the separation of antigens. These disturbances are usually due to a difference in electroendosmosis between the two types of gel. For such a difference gives rise to either a buffer flow between the gels or to dehydration of one of the gels with the dehydration starting at the edge in contact with the other gel. Both phenomena will, of course, severally disturb migration of the antigens into the antibody-containing agarose gel and can even completely prevent it if the two gels lose contact. Which of the two

phenomena will disturb the contact between two gels of different electroendosmosis depends on which gel has the stronger electroendosmosis and on the direction of the electric field (V). To avoid such electroendosmotic disturbances the electroendosmosis of the antibody-containing agarose gel should be adjusted to equal that of the gel strip containing the separated antigens. Several methods are available for such adjustment. The original, weakly cathodal electroendosmosis of commercial agarose, which varies with the brand and batch of the agarose, can be increased by the addition of agar. It can be diminished to zero or be changed to anodal endosmosis by addition of an agarose-derivative with strong anodal electroendosmosis (V). The original cathodal electroendosmosis in agarose can also be diminished by the chemical or chromatographic removal of some of the electroendosmosis-producing negatively charged substituents of the agarose (51,61,83), or by the addition of certain water-soluble polymers, such as methylcellulose (51,81).

The reason why only agarose gels are acceptable for the immunoprecipitation phase in crossed immunoelectrophoresis seems to be the minimal molecular sieving effect of these gels. For if a gel with molecular sieving properties is used the precipitates will appear as diffuse areas probably because the small pores of the gel trap the small soluble antigen-antibody complexes formed in antigen excess (51,52).

9. Buffer system

Good results of crossed immunoelectrophoresis require that the pH and ionic strength of the buffer in the antibody-containing gel be such that the antibodies will be immobile and form immunoprecipitates. No such demands need be placed on the buffer used in the separation of antigens. Such a buffer should, however, not be so concentrated as

significantly to alter the pH or the ionic strength of the antibody-containing agarose gel since such a change can impair the quality of the results. Neither should such a buffer contain solutes capable of forming precipitates by interaction with solutes in the antibody-containing gel since such precipitates may be misinterpreted as immunoprecipitates.

10. Visualization of the pattern of immunoprecipitation

The precipitation pattern obtained on crossed immunoelectrophoresis is visualized with methods generally used for demonstration of immunoprecipitates (direct inspection under oblique illumination or after staining, the use of enzyme-linked or radioactively labelled immunoreactants to bring out faint precipitates). Visualization offers no problems peculiar to crossed immunoelectrophoresis and is therefore not discussed in this thesis. It should, however, be mentioned that the bulk of the proteins not participating in the formation of immunoprecipitates in crossed immunoelectrophoresis migrates off the gel owing to the electric field and thereby often eliminates the time-consuming washing necessary in some methods for visualization of precipitates.

PART THREE

IMMUNOGLOBULINS AS ANTIGENS IN CROSSED IMMUNO- ELECTROPHORESIS

A large number of antigens have successfully been studied with crossed immunoelectrophoresis (47,48,49). The studies have concerned the microheterogeneity and the hereditary polymorphism of the antigens as well as their fragmentation and participation in the formation of complexes. The immunoglobulin classes are examples of extremely heterogenous antigen-populations and several immunoglobulin molecules form complexes with other molecules, in their antibody-function, for example. Crossed immunoelectrophoresis should therefore be a particularly useful method for studying immunoglobulins. But it has not yet been possible to use the method to any notable extent for such studies because good results require a substantial difference in electrophoretic mobility between the antigens and the antibodies *i.e.* a difference generally not existing between immunoglobulins and their corresponding antibodies. The method has, however, been used in some special cases where a small difference in electrophoretic mobility between an immunoglobulin and its corresponding antibodies existed, although the resolving power of the technique was then often low (64,80,84,85,86). This part of the thesis reports how a substantial difference in electrophoretic mobility between an immunoglobulin and its corresponding antibodies can be created and thereby enables the design of a general method for crossed immunoelectrophoresis of immunoglobulins. This part deals also with the interpretation of the precipitation patterns formed on crossed immunoelectrophoresis of immunoglobulins.

Creation of a difference in electrophoretic mobility between an immunoglobulin and its corresponding antibodies

If a difference in electrophoretic mobility between an immunoglobulin and its corresponding antibodies cannot be obtained by choice of pH or by choice of the animal species used for raising the antiserum the difference must be produced by chemical modification of charged groups either in the immunoglobulin or in its corresponding antibodies. Modification of charged groups of the immunoglobulin to be studied by crossed immunoelectrophoresis is unsuitable for the following reasons. Fractionation of the immunoglobulin by zone-electrophoresis or isoelectric focusing will be grossly altered after a chemical modification of its charged groups. The antigenic properties of the immunoglobulin can be altered and individual subpopulations of the immunoglobulin can be affected differently by the modification. Chemical modification should therefore preferably concern only the antibodies against the immunoglobulin.

An ideal chemical modification of charged groups in an antibody-population should produce the intended change of the isoelectric point of the population without significantly increasing the range of isoelectric points of the individual antibodies. Furthermore, it should not change the specificity or notably lower the titer of the antibodies. Two procedures filling most of the above requirements have hitherto been described (70,VI,VII).

One of the procedures is based on the conversion of amino groups of the antibodies to carbamylamino groups by a reaction with cyanate and with a resulting decrease in the isoelectric points of the antibodies. Such antibodies have not yet been used to make crossed immunoelectrophoresis of immunoglobulins generally feasible, but they can probably be used for this purpose, it having been proved that they can be used for electroim-

munoassay of IgG (70).

The other procedure utilizes an activation of carboxyl groups in the antibodies by a carbodiimide and the following reaction of the activated groups with a methylammonium salt with a resulting increase in the isoelectric points of the antibodies (VI,VII). Such antibodies have proved suitable for crossed immunoelectrophoresis of immunoglobulins as well as for electroimmunoassay of immunoglobulins (VI,VII).

Since several procedures for modifying (charged) groups in proteins have been described (87,88) it is highly probable that also procedures other than the two described above can be used for preparing antibody populations suitable for use in crossed immunoelectrophoresis of immunoglobulins.

Choice of a gel-buffer system in which all molecules of an immunoglobulin migrate in the same direction and in which the antibodies against the immunoglobulin are immobile

If the isoelectric point of an antibody population differs notably from that of the immunoglobulin population against which the antibodies are directed, the only further requirement for the creation of a technique for crossed immunoelectrophoresis of the immunoglobulin is a gel-buffer system in which the antibody population is essentially immobile. Such a gel-buffer system is obtained by combining an agarose gel with suitable electroendosmosis and a buffer of suitable pH. The adjustment of the electroendosmosis of an agarose gel has been described in part two of this thesis and will therefore not be considered here. It should, however, be mentioned that agarose gels with high cathodal electroendosmosis contain many negatively charged substituents and therefore sometimes show unspecific adsorption of immunoglobulins (44). This makes such gels unsuitable for use in

crossed immunoelectrophoresis of immunoglobulins. The pH of the buffer can be allowed to vary between 4.5 and 10.3, immunoprecipitation on crossed immunoelectrophoresis or electroimmunoassay having been described in this interval (70,VI,VII). Whether buffers of even more extreme pH-values can be used remains to be tested.

A procedure for testing whether an antibody population is essentially immobile in a gel-buffer system is described (II) as well as procedures for checking that all the molecules of a given immunoglobulin migrate in the same direction in the gel-buffer system used (VI).

Interpretation of the precipitation patterns formed on crossed immunoelectrophoresis of immunoglobulins

Quantitative interpretation of the precipitation pattern obtained on crossed immunoelectrophoresis of an immunoglobulin is more complicated than that of most other proteins (48). This is because of the heterogeneity of the electrophoretic mobility, molecular size and set of antigenic determinants *etc.* of an immunoglobulin.

The differences in the set of antigenic determinants between different subpopulations of an immunoglobulin mean that most antisera against the immunoglobulin react differently with the different subpopulations. This means a source of error in probably all quantitative immunochemical methods, including crossed immunoelectrophoresis. The magnitude of the error of crossed immunoelectrophoresis has not yet been investigated but it probably varies with the type of antiserum and the composition of the immunoglobulin population in the way outlined in part one of this thesis.

An immunoglobulin population contains molecules migrating faster or slower than the bulk of the molecules during the immunoelectrophoretic phase of crossed immunoelectrophoresis. The precipitation pattern obtained

on crossed immunoelectrophoresis of an immunoglobulin will probably somewhat overestimate the concentration of the fast molecules and somewhat underestimate the concentration of the slow molecules. The magnitude of the errors has not yet been studied but it will probably be small as long as the absolute mobility of all different immunoglobulin molecules is high.

The molecules of an immunoglobulin population often differ in size. The precipitation pattern obtained on crossed immunoelectrophoresis overestimates the concentrations of the smallest molecules (VI) and probably underestimates the concentrations of the largest molecules, but the magnitude of such errors has yet to be determined.

Qualitative interpretation of the precipitation pattern obtained on crossed immunoelectrophoresis of an immunoglobulin is not more complicated than that of most other proteins and is therefore not treated in detail here (48). But since immunoglobulins so often take part in the formation of complexes (*e.g.* in their antibody-function) it might not be out of place to give a brief outline of the ways in which such complex formation can influence the precipitation pattern obtained on crossed immunoelectrophoresis. If part of an immunoglobulin population produces a peak on crossed immunoelectrophoresis this peak can be influenced in one of the ways described below if the molecules forming the peak participate in the formation of a complex. 1. The peak diminishes (or disappears) and a new peak appears simultaneously in another position in the precipitation pattern. The morphology of the new peak is often different from that of the original peak (VII). 2. The peak diminishes (or disappears) without the appearance of a new peak. This occurs if the complex formed is insoluble, for example. 3. The peak changes in morphology but not in position or size. 4. The peak does not change

in position, size or morphology despite the formation of a complex. This occurs rarely but it may do so if a very large molecule forms a complex with a very small one (89,90). In such cases demonstration of the formation of a complex requires the use of such procedures as autoradiography (89).

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